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SHORT COMMUNICATION

Salivary nerve growth factor response to intense stress: Effect of sex and body mass index

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Summary Ample evidence links stress to psychiatric and neurological disease. Although many studies examine stress hormone secretion and receptor activity, exciting new developments signify a shift in focus to neuromodulatory systems influencing neuronal development, survival, and neuroplasticity. The purpose of this study was to characterize salivary nerve growth factor (sNGF) responses to intense stress exposure in healthy military members undergoing survival training. A second purpose was to explore effects of age, sex, education, and body mass index (BMI). One hundred sixteen military members (80% male) were studied before, during, and 24 h after a stressful mock-captivity exercise. sNGF was measured at all three time points. Reactivity, recovery, and residual elevation of sNGF were computed. General linear modeling with repeated measures evaluated effect of stress exposure, as well as the roles of age, sex, education, and BMI. sNGF increased 137% from baseline to intense stress. During recovery, sNGF remained elevated an average of 67% above baseline (i.e., residual elevation). Men showed greater sNGF reactivity than women quantified by larger absolute T1 − T2Δ (+148.1 pg/mL vs. +64.9 pg/mL, $p < 0.017$). A noteworthy trend of higher sNGF concentrations in low BMI participants was observed ($p = 0.058$). No effects of age or education were shown. This study shows substantial reactivity and residual

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elevation of sNGF in response to intense stress exposure in healthy humans. Further research is needed to refine the sNGF assay, fully characterize the sNGF stress response, delineate correlates and mechanisms, and validate therapeutic applications.
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1. Introduction

Ample evidence links stress to psychiatric and neurological disease (see Cohen et al., 1995; McEwen, 2012). At the same time, the fact that stress exposure does not inevitably lead to disorder points to individual differences in stress resilience with important prevention and therapeutic implications. A better understanding of how the stress response operates across multiple physiological systems and determinants of response variability could help to explain differential vulnerability to stress-related diseases by sex, body composition, and other known risk factors. For example, women are known to be at greater risk for affective disorders than men, a difference thought to relate to sex-specific biological responses to stress (e.g., Ter Horst et al., 2009). However, a definitive answer to the question of how men's and women's stress responses influence disease susceptibility remains elusive, perhaps because of limitations in the scope of physiological stress assessment (typically, hypothalamic—pituitary—adrenal and/or autonomic nervous system activity).

Although many studies examine stress hormone secretion and receptor activity, exciting new developments signify a shift in focus to neuromodulatory systems influencing neuronal development, survival, and neuroplasticity. In particular, nerve growth factor measured in human saliva (sNGF) has recently been shown to respond to acute stress, offering evidence of a neurotrophic stress-responsive system (Laurent et al., 2013). Further, there is evidence that a dynamic sNGF response to interpersonal conflict stress—i.e., increased reactivity and post-stress recovery—relates to superior psychological adjustment as indexed by stress-related affect and general well-being (Laurent et al., in press; H.K. Laurent, S.L. Laurent, and D.A. Granger, unpublished observations). Some research has documented effects of acute stress on (blood) NGF (Aloe et al., 1994) while other work suggests no effect (Lang et al., 2004). Still other studies have linked reduced blood NGF to stress-related psychiatric illness (see Cirulli and Alleva, 2009). Combined, this work suggests neurotrophic responses could represent a resilience factor that protects certain individuals from adverse effects of stress. Most studies of stress, including the few existing studies of sNGF, involve mild to moderate challenges encountered in daily life. While these provide an important basis for speculation about factors promoting resilience during intense or extreme challenges, further investigation under these more extreme conditions is critical to confirm or refute theories of adaptive stress responses. Convergent findings from mild/moderate and severe stress research would increase confidence that the processes identified in the former apply to conditions such as posttraumatic stress disorder. Thus, an examination of how sNGF responds to severe stress and determinants of differential response could begin to fill important gaps in our knowledge of the basis for health and disease.

The purpose of this study was to characterize sNGF responses to intense stress exposure in healthy military members undergoing survival training. A second purpose was to explore effects of sex, body mass index (BMI), age, and education.

2. Methods

2.1. Military survival training

Survival, Evasion, Resistance, and Escape (SERE) training has been described in earlier reports, (Morgan et al., 2004; Taylor et al., 2012). US military members who are deemed ''high risk of capture'' are required to attend this course, which includes a period of mock captivity. After an initial phase of classroom-based didactic training (5 days), students are taken to a field site where they are trained in SERE techniques (7 days). Training tasks include evasion from a simulated enemy and, upon eventual ''capture,'' students must practice resistance to various forms of simulated exploitation in stressful mock-captivity training challenges. The entire course lasts 12 days, including 1 debrief day after the conclusion of mock captivity.

2.2. Inclusion, exclusion, and compliance criteria

Subjects met inclusion criteria if they were active-duty military members enrolled in SERE training at the Center for Security Forces, SERE Learning Site West (San Diego, California), as part of their military duties and were deemed healthy as indicated by a medical records review conducted by the SERE medical officer. Additional exclusion criteria imposed for this study included smoking; caffeine dependence, any use of anabolic (e.g., DHEA, growth hormone) or ergogenic substance, drug, or supplement (e.g., creatine monohydrate) within the past 3 months; current antihypertensive medication use (e.g., beta-blockers); and current diagnosis of type 1 or type 2 diabetes, with prescribed medication. Compliance requirements were imposed during baseline and recovery assessments. Specifically, subjects were asked to refrain from alcohol ingestion within 12 h of assessments, major meals within 1 h of assessments, and caffeine ingestion within 30 min of assessments. Compliance during mock captivity was implicitly controlled by the training context.

2.3. Protocol

As part of a larger study evaluating stress and health in survival trainees, 116 military members (80.2% male) participated. Half (50.0%) were college educated and two thirds (65.8%) were Caucasian. Mean (SE, range) age, BMI, and

military experience were 25.4 (0.4, 18–46) years, 24.8 (0.3, 18.7–34.0) kg/m^2 and 4.8 (0.4, 0–15) years, respectively. This protocol was approved by the Naval Health Research Center Institutional Review Board.

Participants completed baseline salivary assessments on the first day of the academic phase of SERE training (Time 1 [T1]; pre-stress). Subsequently, all subjects experienced a rigorous evasion exercise, and then participated in a highly realistic mock-captivity scenario. Assessments were performed again directly after a stressful mock-captivity event (Time 2 [T2]; mock-captivity stress). Finally, approximately 24 h after release from mock captivity (marking completion of field training), assessments were completed a third time (Time 3 [T3]; recovery).

A salivary sample was obtained via the passive drool method (Granger et al., 2007) between 1145 h and 1247 h under baseline, free-living conditions on the first day of academic (classroom) instruction for military survival training. Each subject was asked to rinse his or her mouth with water approximately 10 min prior to sample collection and to avoid the following: brushing teeth prior to collection, using salivary stimulants (e.g., gum, lemon drops), and consuming acidic or high-sugar foods within 20 min prior to collection. After data collection, all samples were immediately placed on dry ice and transferred to Salimetrics, LLC (State College, Pennsylvania) for storage and data processing. Samples were assayed for NGF and cortisol (cortisol was included to confirm and quantify the manipulation effect of survival training). The NGF assay was performed in triplicate using a commercially available enzyme immunoassay kit (Promega NGF Emax immunoassay system Cat.# G7731: Madison, WI) modified for use with saliva. The standard curve measured NGF from 3.9 to 250 pg/mL. The assay has an intra-assay precision of 14.5% and an inter-assay precision of 15.5%. Recovery of NGF added to saliva samples averaged 95.3%. Linearity ranged from 82.3% to 127.2%. Salivary cortisol was assayed in duplicate using a highly sensitive enzyme immunoassay (Salimetrics, LLC, State College, PA). The test uses 25 μl of saliva per determination, has a lower limit of sensitivity of 0.003 μg/dl, standard curve range from 0.012 μg/dL to 3.0 μg/dL, an average intra-assay coefficient of variation of 3.5%, and an average inter-assay coefficient of variation of 5.1%. Method accuracy determined by spike recovery averaged 100.8%, and linearity determined by serial dilution averaged 91.7%.

2.4. Statistical analyses

Data were analyzed using SPSS software version 19.0 (IBM, Armonk, New York). Distribution characteristics for all continuous variables were examined to determine if assumptions of normality were met, following conservative predefined limits (e.g., skewness between −1 and 1 (Leech et al., 2005), kurtosis between −3 and 3). Variables exceeding any of these limits were log-transformed prior to performing the relevant statistical test. All data transformations reduced skewness and kurtosis to acceptable levels. Untransformed means are reported for ease of interpretation. Descriptive analyses were conducted to summarize subject characteristics, and independent *t* tests or chi-square tests compared males and females on demographic and background characteristics. For each hypothesis test, a theoretically relevant variable was selected as a covariate if it associated with the independent variable and the endpoint of interest, thus qualifying as a potential confounder (MacKinnon et al., 2000). Separate 2 (group) × 3 (time) analysis of variance or analysis of covariance (ANCOVA) with repeated measures evaluated effects of sex, BMI, age, and education differences across time. Greenhouse–Geisser corrections were implemented when sphericity assumptions were not met. Post hoc independent *t* tests or univariate ANCOVA decomposed the observed group effects at each time point, while post hoc paired *t* tests decomposed the overall time effects. Absolute (value 2 − value 1) and relative Δ scores ([value 2 − value 1/ value 1] × 100%) were also computed and then compared across groups via independent *t*-test or univariate ANCOVA. These calculations were used to operationally define "reactivity" (i.e., initial response from baseline to mock-captivity stress), "recovery" (i.e., change from mock-captivity stress to 24-h recovery), and "residual elevation," (i.e., sustained disruption from baseline to 24-h recovery). Individuals with ≤0% relative T1 − T2Δ were classified as non-responders. All formal hypothesis tests were two-sided, and the probability of committing a Type I error was set at .05. Bonferroni corrections were implemented for each family of group, time series, and delta comparisons (absolute and relative) at .05/3 = .017. Effect sizes were estimated via partial eta-squared (η_p^2; Richardson, 2011).

3. Results

3.1. Nerve growth factor response to intense stress

As shown in Fig. 1, exposure to mock captivity substantially affected sNGF concentrations ($F(2,230) = 58.4$, $p < .001$, $\eta_p^2 = 0.34$). Bonferroni-corrected post hoc comparisons confirmed distinct differences between T1 and T2 ($t(115) = -10.5$, $p < .017$); T2 and T3 ($t(115) = 5.8$, $p < .017$); as well as T1 and T3 ($t(115) = -5.2$, $p < .017$). On average, sNGF increased 136.9% (SE = 15.5%) from baseline to intense stress. During recovery, sNGF remained elevated an average of 67.4% (SE = 11.7%) above baseline (i.e., residual elevation). There were 17 non-responders

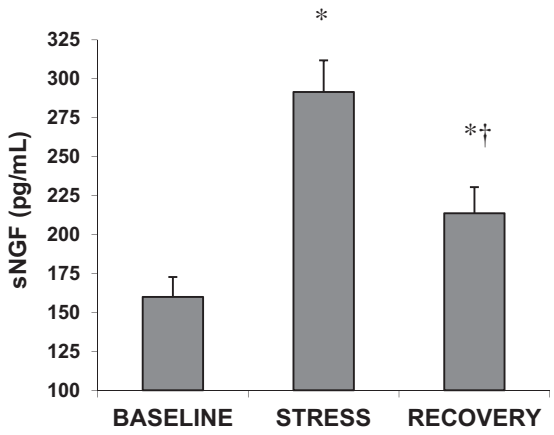

Figure 1 Effect of intense stress exposure on salivary nerve growth factor. *Different from baseline, $p < 0.017$. †Different from stress, $p < 0.017$.

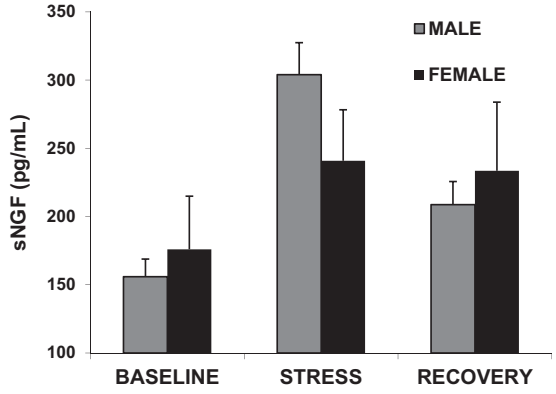

Figure 2 Sex differences in salivary nerve growth factor throughout stress exposure.

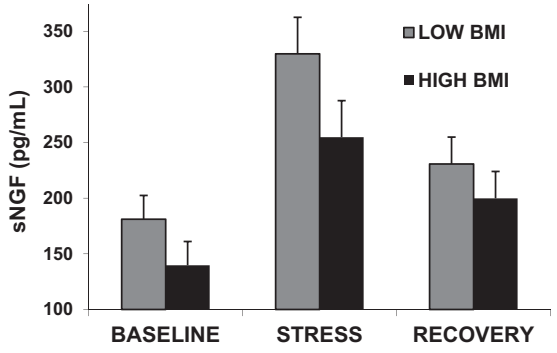

Figure 3 Effect of body mass index (BMI) on salivary nerve growth factor stress trajectory. BMI was dichotomized via median split (cut point = 24.42; group main effect $p = 0.058$). To explore robustness, BMI was dichotomized a second time based on National Institutes of Health (1998) definition of overweight (cut point = 25.0), which yielded a similar effect ($p = 0.105$).

(14.7%). Mean (SE) age, BMI and military experience of nonresponders were 24.9 (1.3), 24.7 (0.4), and 4.5 (4.2), respectively. Of the nonresponders, 13 (76.5%) were male and 8 (47.1%) were college educated.

3.2. Cortisol response to intense stress

Exposure to mock captivity profoundly affected cortisol concentrations ($F(2,228) = 264.4$, $p < .001$, $\eta_p^2 = 0.70$). Bonferroni-corrected post hoc comparisons confirmed sizeable differences between T1 and T2 ($t(115) = -16.9$, $p < .017$); T2 and T3 ($t(114) = 21.2$, $p < .017$); as well as T1 and T3 ($t(114) = 5.2$, $p < .017$). On average, cortisol increased 268.2% (SE = 25.0%) from baseline to intense stress. It fully recovered at T3, decreasing an average of 7.5% (SE = 8.3%) below baseline. Weak associations were noted between salivary cortisol and sNGF, both during intense stress (T2; $r(114) = 0.22$, $p = .02$) and with respect to total hormone outputs across all three time points as determined by area under the curve with respect to ground, ($r(113) = .21$, $p = .02$).

3.3. Effect of sex, body mass index, age, and education

Although males and females did not differ in NGF concentrations at baseline ($p = .82$), males showed greater sNGF reactivity than females, quantified by larger absolute T1 − T2Δ (+148.1 pg/mL [SE = 17.4] vs. +64.9 pg/mL [SE = 20.6], $p < 0.017$; see Fig. 2). A noteworthy trend of higher overall sNGF concentrations in low BMI participants was observed (main effect: $F(1,104) = 3.7$, $p = .058$, $\eta_p^2 = 0.03$; see Fig. 3). Neither age ($p = .46$) nor education ($p = .57$) associated with the sNGF trajectory. The salivary cortisol response trajectory was not influenced by sex, BMI, age, or education (all $p > .05$). Small sample size precluded formal investigation of non-responder status by sex, BMI, age, or education.

4. Discussion

This study is the first to our knowledge to show a neurotrophic response to extreme stress in humans, building on previous findings involving moderate stressors. In particular, we found a significant sNGF response to mock captivity in military personnel, as well as individual differences related to sex and BMI. This work sheds further light on a neurotrophic stress-responsive system that could help to explain important links between stress and health.

The marked stress-related rise in sNGF and incomplete post-stress recovery are consistent with sNGF findings in other samples, which have shown non-recovery or a delayed rebound following stress (Laurent et al., in press; Laurent et al., 2013; H.K. Laurent, L. Stroud, B. Brush, and D.A. Granger, unpublished observations). At the same time, the magnitude of the sNGF rise was greater than that detected previously, suggesting a dose-response effect by which higher stress severity elicits higher neurotrophic elevation. The current findings are also consistent with Aloe et al. (1994) findings linking acute stress to plasma NGF concentrations (Aloe et al., 1994) in novice military parachute jumpers, yet inconsistent with Lang et al. (2004) work suggesting no effect of an academic oral presentation on serum NGF of healthy male volunteers. Further investigation of sNGF during and after a range of stressors with longer follow-up periods will be necessary to clarify possible dose-response effects and typical recovery times. The adaptive value of post-stress neurotrophic elevations should also be explored.

Differences in sNGF by sex and BMI could help in understanding differential health vulnerabilities associated with these factors. Given known links between neurotrophin levels and mood disorder, women's blunted neurotrophic responses to severe stress may play a role in the well documented sex imbalance in these disorders. In particular, the failure to launch a robust neurotrophic response during stress could leave central and peripheral neural systems vulnerable to stress-related damage, which in turn could lead to negative mental health sequelae. Similarly, part of the health costs associated with high BMI may be attributable to blunted neurotrophin levels; NGF is known to regulate endocrine and immune function and to provide protection from inflammatory responses (Colafrancesco and Villoslada, 2011). It is also possible that reduced neurotrophin levels represent a downstream effect of physiological changes related to increasing BMI. These findings should be followed up with research that

can address associations with mental and physical health outcomes, as well as directionality of effects.

Limitations of this study should be recognized. Although mock-captivity is a very realistic and ecologically valid psychosocial stressor, it is known to have physical and physiologically stressful elements; thus it is best described as a ''composite'' stressor. Also, sex hormones are believed to influence neurotrophin activity, but were not controlled in this study. Futhermore, it is noted that several sNGF sample absorbance values fell outside the curve linearity. Some non-NGF material, then, may have been present in saliva after stress which could increase the unspecific binding and, as a result, overestimate stress-induced NGF concentrations. Although the assay used to estimate sNGF reflected current state of the art, it is currently undergoing refinement. Finally, mental/physical health was not assessed in this study. In future work, predictors of neurotrophic response to differing stressors should be related to relevant health outcomes over time to clarify the role of sNGF in stress-health paths. Also, research in larger and more diverse samples will permit closer analysis of non-responders and provide recommendations for possible intervention. Finally, although assessing neurotrophin levels under severe stress conditions represents an important step toward discerning real-world stress effects, it would be informative to compare magnitudes of response and effects on well-being across different types and severities of stress. For now, this study confirms that the human neurotrophic system responds to extreme stress, but that the nature of this response varies across individuals. Combined with findings under moderate stress conditions, this work highlights the importance of stress-responsive neurotrophins for understanding resilience.

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Conflicts of interest

DAG is founder and Chief Strategy and Scientific Advisor at Salimetrics LLC (State College, PA). DAG's relationship with this entity is managed by the policies of the Conflict of Interest Committee at the Johns Hopkins University and the Office of Research Integrity and Assurance at Arizona State University.

All other authors declare that they have no conflicts of interest.

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14. ABSTRACT

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15. SUBJECT TERMS
stress, nerve growth factor, plasticity, military, survival, sex differences, body mass index

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